Continuous Release of bFGF from Multilayer Nanofilm to Maintain Undifferentiated Human iPS Cell Cultures

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Experimental Section

Human iPS cells and cell culture A human iPS cell line was investigated in this study. This cell line was generated by introducing four transcription factors (Oct4, Sox2, cMyc, and Klf4) into human skin fibroblasts. The four factors were introduced using an inducible lentivirus system. The human iPS cell line was maintained on mitomycin C-treated mouse embryonic fibroblasts (MEFs) in DMEM supplemented with 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 1 mM β-mercaptoethanol, 1× non-essential amino acids (NEAA; Invitrogen), 20% knock-out serum replacement (KSR; Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 25 ng/mL bFGF (R&D Systems, Minneapolis, MN, USA).

Immunostaining assay Cells were fixed with 4% paraformaldehyde for 20 min at room temperature and washed in phosphate-buffered saline (PBS). The cells were then incubated with primary antibodies to Oct4 and SSEA-4 diluted in PBS containing 10% goat serum and 0.1% Triton X-100 overnight at 4°C. Following the incubation, the construct was washed five times in PBS. Then, the cells were incubated in a 1:500 dilution of Alexa 488-labeled secondary antibody for 1 h at room temperature. Finally, the construct was counterstained with DAPI and analyzed by fluorescence microscopy.

Quantitative alkaline phosphatase activity assay and western blotting To generate iPS cells, iPS cell colonies were incubated with 0.1% collagenase solution for 20 min and then detached with gentle pipetting. To remove the MEF feeder cells, iPS cells were allowed to settle down in a 15-mL conical tube for 10 min and the supernatant containing the MEFs was removed. The pellet was collected and completely dissociated in PBS by vigorous pipetting. Total protein content was determined using BCA protein assay kit. Cell extracts were prepared with RIPA buffer (50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktail) at 4°C for 1 h followed by centrifugation, and the total protein content of the lysate was measured according to the manufacturer's protocol. For quantitative analysis of alkaline phosphatase (AP) activity, cells were resuspended in p-nitrophenyl phosphate (p-NPP) buffer. Then, p-NPP substrate solution was added, and stop solution was added after 20 min. The absorbance was measured at 405 nm using a microplate reader. To determine AP activity per the same number of cells, the absorbance was normalized to the total protein. For western blot analysis, iPS cell extracts containing equal amounts of total protein were resolved by SDS-PAGE. The blotted membranes were incubated with primary antibodies at room temperature for 1 h. The primary antibodies used in this study were anti-Oct4 monoclonal antibody (1:1000; Santa-Cruz Biotechnology, Dallas, TX, USA) and anti-actin monoclonal antibody (1:2000; Santa-Cruz Biotechnology). For detection of primary antibodies, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:2500; Santa-Cruz Biotechnology) at room temperature for 1 h. After several washes, the membranes were developed with an ECL substrate solution (GE Healthcare, Uppsala, Sweden).

Materials Poly-L-lysine (PLL) (MW, 80 kDa) and starch (ST) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and used without further purification. bFGF (17 kDa; pI, 6.1) and bFGF enzyme-linked immunosorbent assay (ELISA) kits were obtained from R&D Systems. Glass slides (for substrates) were obtained from VWR Scientific (Edison, NJ, USA).

Film construction and treatment of prepared films Multilayer films were assembled layer-by-layer (LbL) on a silicon wafer and quartz glass by alternatively dipping the substrate in aqueous solutions using a programmable Carl Zeiss slide stainer at room temperature. First, the silicon wafer and quartz glass were treated with Piranha solution for 10 min (sulfuric acid/hydrogen peroxide (H_2O_2): 70/30 v/v%). Subsequently, negative charge modification of substrates was performed by heating at 70°C for 30 min in a 5:1:1 vol% mixture of water, H_2O_2, and 29% ammonia solution (RCA solution). The substrate was first dipped in a positively charged 1 mg/mL chitosan- or 10 g/mL bFGF-dispersed aqueous solution (pH 4 for PLL, pH 5 for bFGF) for 10 min. After the deposition of each layer, the substrate was thoroughly rinsed three times in pH-adjusted deionized water for 1 min each. Then, the substrate was dipped in a negatively charged 1 mg/mL starch solution (pH 6.0) for 10 min, and the above-described rinsing steps were performed. This dipping process was repeated until a desired number of bilayers was achieved. For starch gelatinization, the prepared multilayer films were treated at 40°C for 20 min.
Human iPS cell were cultured in DMEM supplemented with 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 1 mM β-mercaptoethanol, 1× non-essential amino acids (NEAA; Invitrogen), 20% knock-out serum replacement (KSR; Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 25 ng/mL bFGF (R&D Systems, Minneapolis, MN, USA) coated with the electrostatic films as described. Media was changed and aliquots from culture were frozen down every 48

hours and assayed for specific pluripotency marker proteins according to the manufacturer's protocol. All assays were performed at least three times in triplicate.

Film and release characterization The morphologies of the (PLL/ST/bFGF/ST)n (n = number of tetralayers) multilayer coatings were examined using field emission-scanning electron microscopy (FE-SEM; JEOL JSM-7401F). Film growth was monitored by profilometry (Tekan) at five different predetermined locations on the dried film surface. Release experiments were conducted by immersing a prepared multilayer film into a 10-mL vial containing 2 mL of model physiological solution, PBS (aqueous salt solution containing sodium chloride, sodium phosphate, potassium chloride, and potassium phosphate; the osmolarity and ion concentrations of PBS at 37°C and 5% CO_2. Release profiles were measured using bFGF ELISA.

Figure S1.

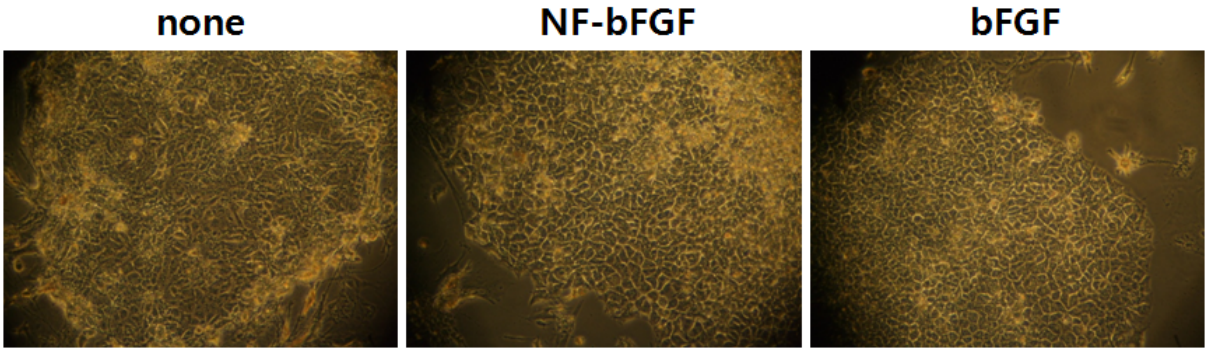

Fig. S1. Effect of NF-bFGF on the morphology of iPS cells. At 70% confluency, human iPS cells were collected by collagenase treatment and subsequently dissociated into small clumps. The clumps were seeded onto MEF feeder layers and cultured with recombinant bFGF, NF-bFGF, or without bFGF. After 6 days of culture, cell morphologies were observed by microscopy. Microscopic images showing the morphologies of the cultured iPS cells.

Figure S2.

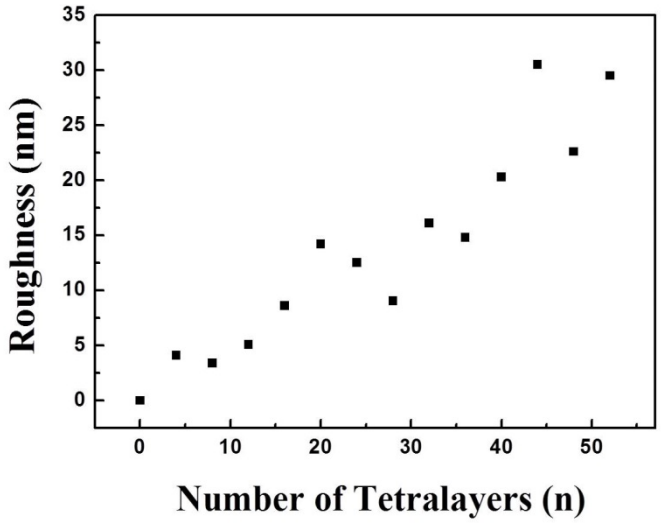

Fig. S2. Roughness of $(PLL/ST/bFGF/ST)_{n\ (n\ =\ number\ of\ tetralayers)}$ multilayer films.